

Active growth factor delivery from poly(D,L-lactide-co-glycolide) foams prepared in supercritical CO₂

David D. Hile, Mary Lee Amirpour, Aydin Akgerman, Michael V. Pishko*

Texas A&M University, Department of Chemical Engineering, College Station, TX 77843-3122, USA

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Abstract

A method for the production of microporous poly(D,L-lactide-co-glycolide) foams containing encapsulated proteins using supercritical carbon dioxide is described. Foams generated as aqueous protein emulsions in a polymer-solvent solution were saturated with carbon dioxide at supercritical conditions, and then suddenly supersaturated at ambient conditions causing bubble nucleation and precipitation of the polymer. Proteins contained in the water phase of the emulsion were encapsulated within the foams, including basic fibroblast growth factor (bFGF), an angiogenic factor of interest in tissue engineering applications. The release and activity of bFGF from these foams was determined in vitro and compared with similar porous scaffolds prepared by traditional solvent casting-salt leaching techniques. Total protein release rate was greater from structures made in CO₂ than those made by the salt leaching technique, however a large initial burst of bFGF was released from the salt leached structures. This initial burst was not observed from the polymer foams processed in CO₂ and active bFGF was released at a relatively constant rate. Residual methylene chloride levels were measured in the foams made with CO₂ and were found to be above the limits imposed by the US Pharmacopoeia implying that further solvent removal would be required prior to in vivo use. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Both natural and synthetic scaffolds for tissue engineering provide a matrix onto which cells may adhere and aid in shaping and defining cell growth in vivo [1–3]. In the past, synthetic scaffolds have been used to form neocartilage in the shape of a human ear, tendons for use in orthopedic surgery and human urothelial and bladder muscle structures [4]. One

problem associated with large, engineered tissue constructs is the neovascularization of the cell/material construct. Neovascularization, or angiogenesis, is the process leading to the formulation of new blood vessels that develop from pre-existing vasculature. This is critical to the success of engineered tissues and transplanted cell-containing devices because blood vessels provide growing cells with oxygen and nutrients necessary for survival. Neovascularization of the tissue construct may be enhanced through the controlled delivery of specific angiogenic growth factors, including basic fibroblast growth factor (bFGF) and vascular endothelial

*Corresponding author. Tel.: +1-409-847-9395; fax: +1-409-845-6446.

E-mail address: pishko@tamu.edu (M.V. Pishko)

growth factor (VEGF), known to promote angiogenesis *in vivo* [5–7]. In addition to acting as matrix structures for tissue growth, these polymeric scaffolds may also act as drug delivery devices for angiogenic factors [8,9]. Incorporating growth factors into the scaffold or foam matrix allows controlled release of the angiogenic factor while maintaining an environment for cell attachment and growth over a period of weeks to months.

A traditional method of polymer scaffold preparation utilizes a solvent casting–salt leaching technique [10–13]. By this procedure, the polymer solution is poured onto a bed of salt particles with defined size. The solvent is evaporated under vacuum causing the polymer to precipitate and solidify around the salt particles. The salt is then leached out of the scaffold, without altering the pore structure of the polymer, by numerous rinses in distilled water. The use of salt particles results in a defined pore size for the scaffold. The introduction of angiogenic factors emulsified within the polymer solution prior to the solvent casting–salt leaching process makes it possible to entrap these factors within the polymer scaffold. As the organic solvent is removed, the polymer precipitates and encapsulates the angiogenic factors.

Porous polymeric matrices have alternatively been prepared with carbon dioxide [13–16]. This technique involves lowering the glass transition temperature of the polymer under high-pressure CO_2 by increasing the number of gas molecules absorbed into the polymer phase. Upon depressurization, the polymer is suddenly supersaturated with CO_2 causing bubbles to form and grow within the polymer as two phases develop [17]. As the glass transition temperature of the polymer increases following depressurization, the pores generated by CO_2 bubble nucleation become permanent creating a microporous foam.

The focus of this study was to compare the *in vitro* release of proteins and activity of a useful angiogenic factor, bFGF, from foams prepared in supercritical CO_2 (SC- CO_2) and scaffolds produced by the solvent casting–salt leaching technique. In particular, the influence of processing in SC- CO_2 on protein release kinetics and activity of an angiogenic factor, bFGF, was investigated.

2. Materials and methods

2.1. Materials

Anhydrous carbon dioxide of 99.8% purity was purchased from Bailey Oxygen and Tool Company (Bryan, TX, USA). Microporous foams were prepared from copolymers of lactide and glycolide. Poly(D,L-lactide-co-glycolide) with a lactide-to-glycolide ratio of 80:20 (PLGA 80:20) was obtained from Polysciences (Warrington, PA, USA). The polymer was processed as received and had a molecular mass of 18 000 as determined by size-exclusion chromatography. PLGA 65:35 with a molecular mass of 52 000 was obtained from Aldrich (Milwaukee, WI, USA). Methylene chloride purchased from Fisher Scientific (Pittsburgh, PA, USA) was used as the polymer solvent. Salt for use in the solvent casting–salt leaching scaffold preparation technique was purchased from Fisher Scientific.

Bovine serum albumin (BSA), used as a model protein to investigate drug encapsulation within the porous polymer matrix, was obtained from Sigma (St. Louis, MO, USA). Total protein concentrations were measured with Coomassie Protein Assay Reagent purchased from Pierce (Rockford, IL, USA). The buffer used during the protein release studies consisted of 0.1 M phosphate-buffered saline (PBS) at a pH of 7.4 containing 1% antibiotic/antimycotic from Sigma to prevent bacterial growth. The composition of PBS used in this study was 0.144 g/l KH_2PO_4 , 9.00 g/l NaCl (both from Fisher Scientific), and 0.795 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Mallinckrodt, Paris, KY, USA).

Recombinant human bFGF with a molecular mass of 17 400 was obtained from R&D Systems (Minneapolis, MN, USA). The activity of released bFGF was determined by its ability to promote the incorporation of ^3H -thymidine (ICN Pharmaceuticals, Costa Mesa, CA, USA) into mouse-BALB/c fibroblasts received from ATCC (Rockville, MD, USA). Fibroblast cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, both obtained from Sigma. Acetic acid, trichloroacetic acid and NaOH (Fisher Scientific) were used in the active bFGF assay.

2.2. Scaffolds prepared by salt-leaching

PLGA scaffolds containing encapsulated proteins were manufactured using a combination of published salt casting [10–12] and emulsion techniques [18]. An aqueous phase consisting of 1.25 μg bFGF and 10 mg BSA dissolved in 250 μl of 0.1 M PBS at pH 7.4 was prepared. The aqueous phase was added to a 3% (w/v) solution of PLGA–methylene chloride containing 0.5 g polymer and sonicated on a Branson Model 450 Digital Sonifier equipped with a tapered microtip (1/8 in. diameter) at 30% amplitude for 2.0 s to form a homogenous emulsion (1 in.=2.54 cm). This emulsion was then poured onto a bed of sieved sodium chloride particles with a size of 250 μm or larger. The scaffolds were placed under vacuum in a dessicator for 24 h, and then were repeatedly washed with copious amounts of distilled water to leach the salt.

2.3. Foams prepared in SC- CO_2

Prior to SC- CO_2 processing, an emulsion was prepared consisting of an aqueous protein phase and an organic polymer solution phase. The organic

phase was made by dissolving 1 g of PLGA into 2.0 ml of methylene chloride, and the aqueous phase consisted of 1.25 μg of bFGF and 20 mg BSA dissolved in 200 μl of 0.1 M PBS at pH 7.4. A homogenous water-in-solvent emulsion was prepared by sonicating the immiscible organic and aqueous phases for 2.0 s on the Branson Sonifier. A mold (D in Fig. 1) made from a 1.5 \times 1 in. 316-stainless steel cylinder with a 7/16 in. bore was filled with 1.0 ml of the water-in-solvent emulsion. The cylindrical mold was sectioned longitudinally so that the halves could easily be separated following SC- CO_2 processing to remove the polymer foam. A PTFE gasket was used as a seal for the mold. The mold and PTFE gasket were constructed on site.

Immediately after the emulsion was pipetted into the mold, the mold was placed into a 316-stainless steel pressure cell (F) constructed from a 2 \times 2 in. cylinder with a 1.125 in. bore. The pressure vessel was pressurized to 80 bar with CO_2 (A) by a miniPump metering pump, Model No. 870046, from Thermo Separation Products (Riviera Beach, FL, USA) (B). The pressure cell was maintained at 35°C by keeping the cell within a silicone oil bath maintained at constant temperature.

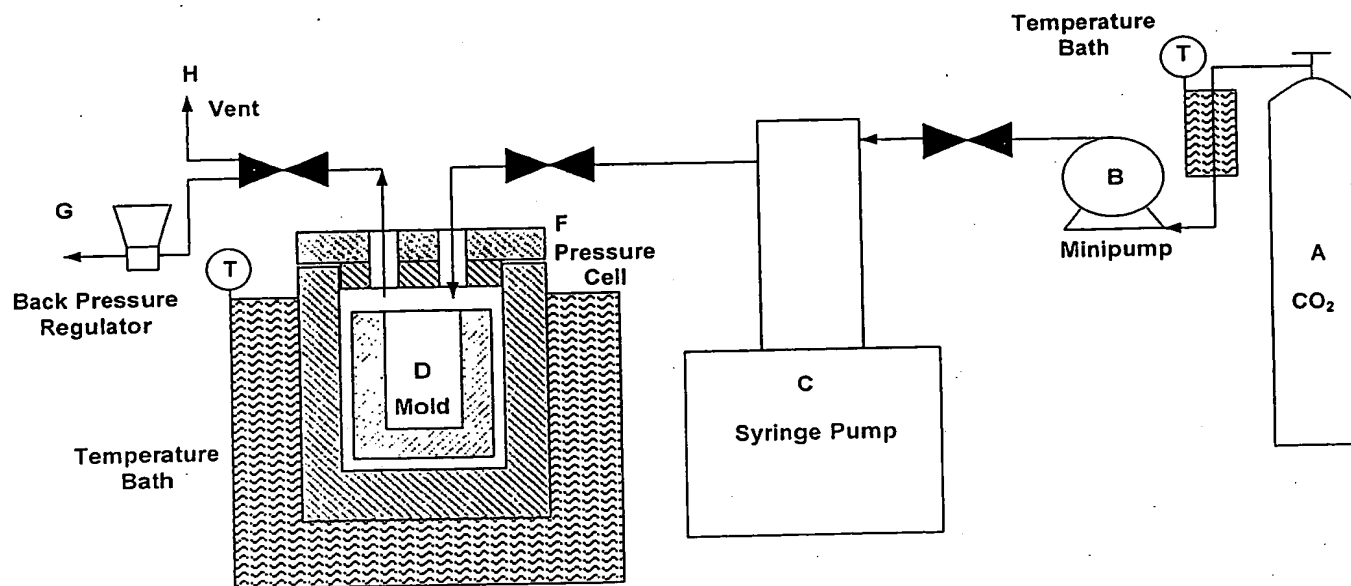


Fig. 1. Schematic of the system used to generate PLGA foams in supercritical carbon dioxide. Methylene chloride was removed and the polymer was foamed in a stainless steel mold (D). Carbon dioxide at a temperature of 35°C and pressure of 80 bar was utilized to extract the organic solvent, saturate the polymer phase, and generate a porous structure upon rapid depressurization.

Once CO₂ had reached the desired operating pressure, a steady 15 ml/h flow-rate of CO₂ was produced through the pressure cell by an LC-5000 Syringe Pump manufactured by Isco (Lincoln, NE, USA). The pressure was controlled within 1 bar of the set point by a back pressure regulator (G), Model No. 26-1722-24-161, obtained from Tescom (Elk River, MN, USA). The pressure was held constant for 24 h in order to extract methylene chloride and saturate the polymer with CO₂. Finally, the pressure cell was depressurized rapidly (typically 10–12 s) by opening a vent valve (H), thus creating a microporous polymer foam.

After the pressurization and rapid depressurization sequence, the porous polymer foam was removed from the mold and sectioned for use in protein release studies. The dry mass of each section (approximately 50 mg) was measured and samples were placed into polypropylene microcentrifuge tubes containing 1.5 ml of PBS. The tubes were incubated at a constant temperature of 37°C, and the PBS buffer was frequently changed. Buffer removed from the tubes was assayed for active bFGF, and the total protein concentration was measured by the Bradford technique [19].

2.4. Scanning electron microscopy (SEM) analysis

Sections of PLGA were also prepared for SEM analysis to study the pore structure and pore size of polymer structures prepared by both CO₂ expansion and salt leaching. The polymer constructs were quick-frozen in liquid nitrogen and sectioned to reveal an intact pore network. The samples were attached to aluminum mounts and coated with 200 Å of gold–palladium. Samples were observed on a T330A scanning electron microscope from JEOL (Peabody, MA, USA). Typical pore structures are displayed in Fig. 2 for polymer processed in SC-CO₂ and via the solvent casting–salt leaching approach.

2.5. bFGF activity

The activity of released bFGF from polymer scaffolds and foams was determined by measuring the uptake of ³H-thymidine in bFGF-stimulated fibroblast cells [20]. The fibroblast cells were seeded onto a 96-well tissue culture plate at a density of

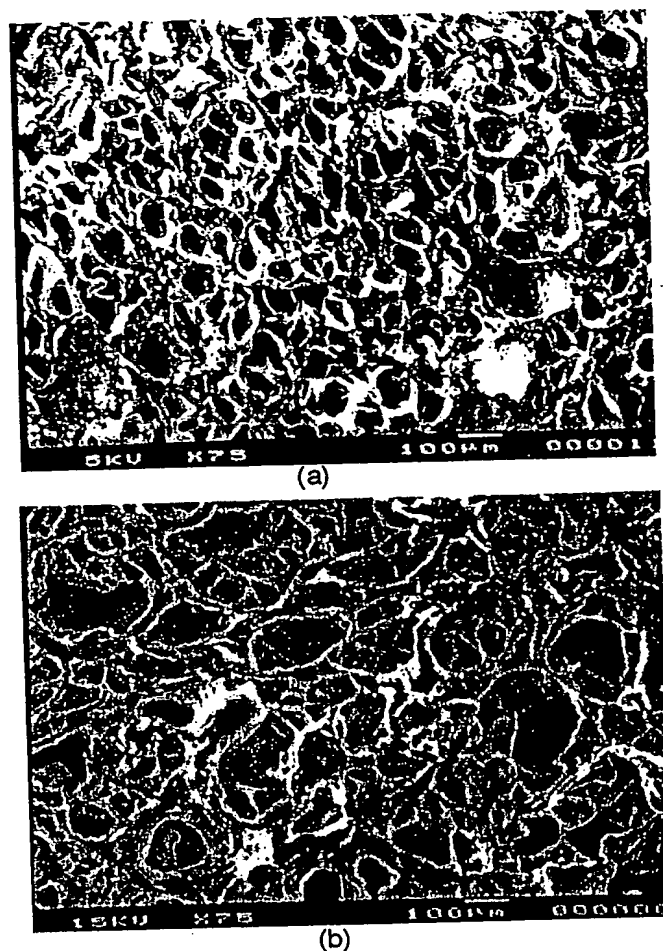


Fig. 2. Scanning electron micrographs of PLGA 65:35 structures. The foam (A) was generated by the rapid expansion of CO₂. The scaffold (B) was prepared by the solvent casting–salt leaching technique.

10 000 cells/well. The cells were incubated with 200 μl of DMEM per well containing 10% fetal bovine serum for 24 h. The media was aspirated and replaced with 200 μl of serum-starved DMEM containing only 0.02% BSA, followed by incubation for 24 h. The serum-starved media was aspirated and replaced with 150 μl serum-starved media containing 0.5 μg heparin per well. After incubation for 1 h, 100 μl PBS containing released bFGF was added to each well. The cells were incubated for 18 h before 50 μl of serum-starved media, containing 0.3 μCi of ³H-thymidine, was added to each well.

The cells were incubated for 24 h following the addition of ³H-thymidine. After 24 h, the media was

aspirated and 200 μ l of ice cold 10% (w/v) trichloroacetic acid (TCA) was added to each well to precipitate the cellular DNA. The TCA was left in the plate for 5 min and was subsequently removed. This step was repeated three times for a total of four TCA washes, after which 50 μ l of 1.0 M NaOH was added to each well and incubated at room temperature for 30 min to rupture the cellular membrane. The NaOH was neutralized with 25 μ l of 2.0 M acetic acid and the extent of thymidine uptake was measured by adding 200 μ l of Microscint 20 liquid scintillation cocktail (Packard, Meriden, CT, USA) to each well and measuring the counts per minute on a Packard TopCount Scintillation Counter (Model B9904). The concentration of unknown bFGF solutions was determined by a standard log bFGF concentration versus counts per minute curve generated from known concentrations of bFGF.

2.6. Residual methylene chloride concentrations

The residual concentration of methylene chloride in the SC-CO₂ processed foams was measured by gas chromatography (GC) with electron-capture detection at Galbraith Labs. (Knoxville, TN, USA).

3. Results and discussion

3.1. Foam generation in SC-CO₂

Porous polymer structures were prepared with SC-CO₂. Carbon dioxide beyond its critical temperature and pressure has enhanced solvent capabilities enabling the biodegradable polymer, PLGA, to be plasticized. The reduction of the glass transition temperature is potentially a result of interactions between carbon dioxide and PLGA leading to increased mobility of ester groups throughout the polymer backbone. Studies of poly(methyl methacrylate) (PMMA) revealed interactions between the lone pair electrons of carbonyl functionalities in PMMA with CO₂ [21] and, thus, similar interactions may exist with PLGA. The enhanced chain mobility increases the free volume of the polymer, thereby producing a greater equilibrium content of gas within the polymer phase.

Upon rapid depressurization, the solvent capabilities of CO₂ are drastically reduced. The polymer phase, which was saturated beyond the critical pressure and temperature of CO₂, becomes super-saturated at ambient conditions. Nucleation of gas bubbles occurs as the reduced pressure gas attempts to escape from the polymer phase. Coinciding with bubble formation, the polymer that had been plasticized by the supercritical fluid begins to vitrify. A microporous foam results as the increase in the glass transition temperature of the polymer "locks in" the porous structure created by the gas nucleation process.

The solvent capabilities of SC-CO₂ may also be utilized to extract solvents from the polymer phase such as methylene chloride from PLGA. A phase separation occurs as the solvent, which is soluble in the surrounding CO₂ phase, is gradually removed, causing the insoluble polymer to precipitate and dry. Since SC-CO₂ is a weak solvent in general, the solvent removal process occurs over the course of several hours and is dependent upon the ratio of solvent to polymer present.

The initial use of a solvent may aid protein encapsulation within the foam. An emulsion of proteins dissolved in an aqueous buffer within polymer dissolved in an organic solvent, formed prior to pressurization, should yield a distribution of proteins throughout the polymer phase following supercritical drying. Encapsulation is achieved as the supercritical fluid removes the solvent to precipitate the polymer and entrap the dispersed proteins.

In this study, SC-CO₂ was utilized both to extract methylene chloride from a 0.1 M PBS-protein emulsion in methylene chloride-PLGA and to foam the dried polymer through rapid expansion of CO₂. The polymer processing time under SC-CO₂ conditions (temperature equal to 35°C and pressure equal to 80 bar) was varied from 10 to 24 h. The optimum processing time was approximately 24 h, as this allowed for sufficient time for methylene chloride removal and CO₂ saturation of the polymer phase. Processing times less than 18 h consistently resulted in amorphous polymer structures that were plasticized by the presence of residual methylene chloride. Processing times beyond 18 h yielded polymer foams with defined pores and a stable pore structure.

3.2. Active proteins released from foams

Pharmaceutical processing techniques in SC-CO₂ have been developed as an alternative to recrystallization in organic solvents, spray drying, milling and lyophilization [22,23]. Advantages of processing in SC-CO₂ include the use of an environmentally benign solvent and the ability to produce powders with a controlled particle size distribution [24]. Protein powders of lysozyme, insulin and trypsin produced via the supercritical antisolvent technique (SAS) exhibited 69% to near 100% retention of biological activity, strongly dependent on the protein [24]. The SAS approach is known to have an effect on the protein's secondary structure with an increased content of β -sheet formation and coinciding decrease in α -helicity [25]. In this study, the potential mechanisms for protein denaturation besides high pressure include incubation in an aqueous buffer at physiological temperatures, unfavorable interactions with organic solvent, heat generated during sonication, and protein-polymer interactions within the polymer foam.

In addition to the possible deactivating conditions involved in encapsulating bFGF within PLGA foams or scaffolds, the inherent instability of bFGF was a concern [26–28]. Loss of activity may be associated with conformational changes within the protein including irreversible oxidation in the form of intramolecular disulfide bonds. Also, bFGF has a strong affinity towards irreversibly binding to glass or plastic surfaces in solution prompting physical loss of the protein [27,29].

The amount of bFGF released was based on the assumption that the ratio of bFGF to BSA released is the same as that loaded into the polymer. The total protein release rate varied between the two polymer types under investigation (Fig. 3). Initially, the release rate of protein from both polymer types was similar implying that the encapsulation efficiency (approximately 97.5%) was reproducible between PLGA 65:35 and PLGA 80:20. However, subsequent protein release rates were faster from foams made from PLGA 65:35 than in foams made from PLGA 80:20. An increase in the glycolide content in the polymer is expected to increase the release rate as described by Batycky et al. [30].

The release rate of active bFGF from both PLGA

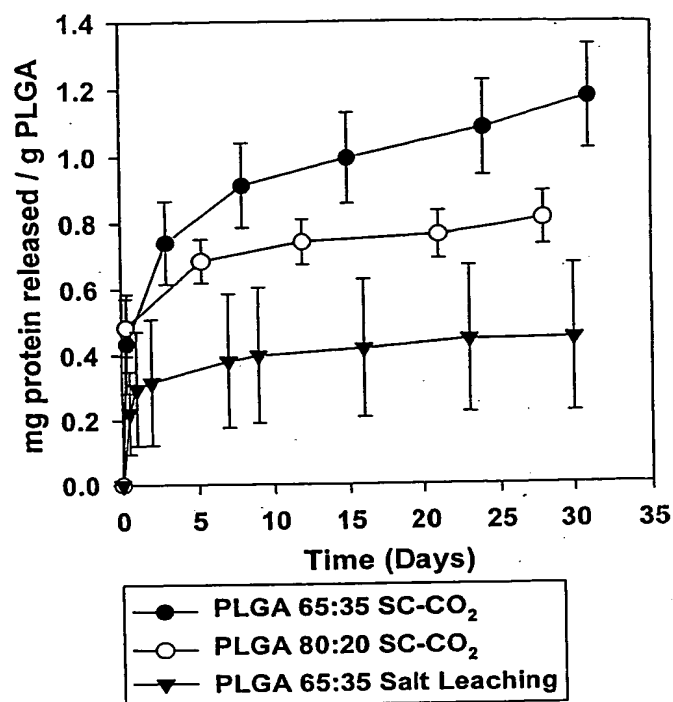


Fig. 3. Total protein released from PLGA structures containing bFGF. Total protein measurements include bFGF and BSA released from PLGA structures. The data represent the average fraction of total protein released during four independent trials with error bars reflecting the standard deviation.

65:35 and PLGA 80:20 foams was relatively constant for 3 weeks (Fig. 4). Differences in the release rate of active bFGF from both polymer types were not distinguishable. As in the case of total protein release, the concentration of active bFGF at the surface of PLGA 65:35 and PLGA 80:20 foams were similar. The amount of active bFGF detected during the first day of the release studies represents only 0.2% of the theoretical maximum value based upon the total amount of protein released, assuming the ratio of BSA to bFGF released remains constant. This percentage increased to 2% after day 1. The overall loss of activity can be attributed to the unstable nature bFGF as described earlier. The influence of SC-CO₂ exposure on stability is not yet known.

3.3. Comparison between foams and scaffolds

Total protein release rates were greater from

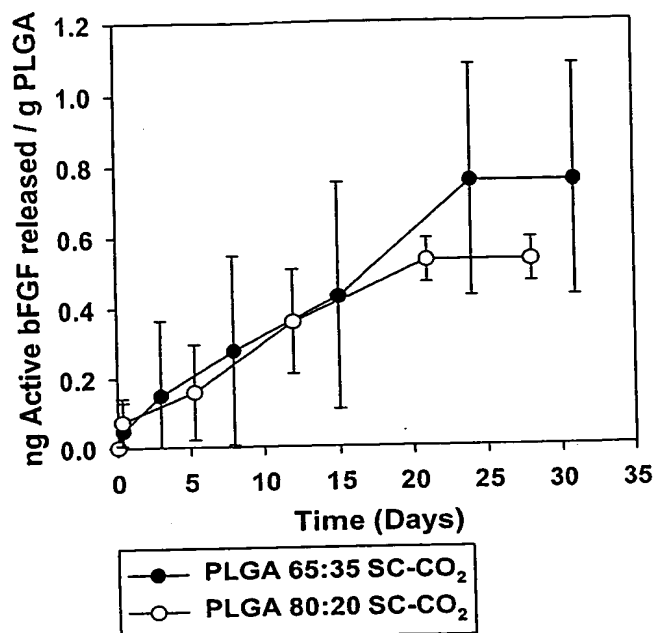


Fig. 4. Release rate of biologically active bFGF from PLGA foams made in SC-CO₂. The data represent the average amount of active bFGF measured during four independent trials with error bars reflecting the standard deviation.

foams generated in SC-CO₂ than by scaffolds made by solvent casting–salt leaching as seen in Fig. 3. Initial protein release from foams processed in CO₂ was approximately twice the rate observed in the salt leached scaffolds. The initial release rate from the scaffolds does not include protein lost during the distilled water salt leaching steps, as protein levels were too dilute for detection. Release rates of encapsulated proteins were also higher from foams generated in SC-CO₂ through 30 days.

Scaffolds prepared by salt leaching demonstrated a large initial burst of active bFGF that was not observed in foams made in SC-CO₂ (Fig. 5). Following this initial burst, the release rate of active bFGF from scaffolds was approximately twice the rate demonstrated for foams. Therefore, it is likely that there was significant deactivation of bFGF through interactions with SC-CO₂ not only at the foam surface, but also for protein encapsulated within the foam.

3.4. Residual solvent analysis

Residual methylene chloride levels in foams pre-

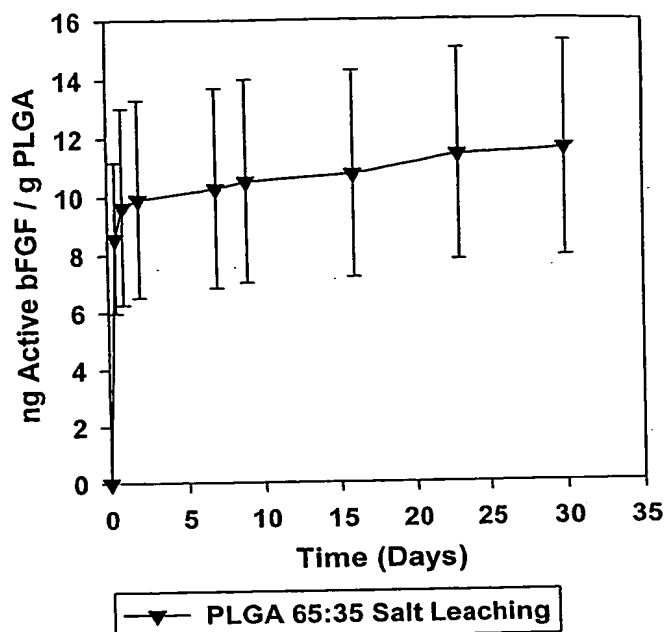


Fig. 5. Release of biologically active bFGF from PLGA scaffolds made by the solvent casting–salt leaching technique. Active bFGF was released from the scaffolds in a large initial burst in comparison to PLGA structures made in SC-CO₂.

pared in SC-CO₂ were determined by GC analysis. A slight difference in residual solvent level was observed between the two polymer types. Methylene chloride was found to be present in PLGA 80:20 at a mass fraction of 0.032, while the fraction found in PLGA 65:35 was determined to be 0.018. Both of these levels are well above the limit of 600 µg/g established for methylene chloride in pharmacopeial articles by the US Pharmacopeia [31]. Additional methylene chloride removal would be required prior to the in vivo use of foams generated by techniques described here. Either longer SC-CO₂ extraction times than used in this study or methylene chloride removal prior to foam generation in CO₂, such as vacuum drying, may significantly reduce final methylene chloride levels in these devices.

4. Conclusions

Porous PLGA foams with encapsulated protein were generated in SC-CO₂ as constructs for tissue engineering applications. SC-CO₂ processed foams

released more protein per gram of polymer than from traditional structures made by solvent casting–salt leaching. The release rate of active bFGF from salt leached scaffolds were greater than those observed from foams suggesting that the growth factor was deactivated by interactions with SC-CO₂. Residual methylene chloride levels in foams prepared in CO₂ were beyond limits imposed by the US Pharmacopeia implying that further solvent removal would be required for these devices prior to in vivo use. However, the ability to encapsulate and release protein at a controlled rate demonstrates the potential to generate porous structures in SC-CO₂ capable of releasing active angiogenic factors for tissue engineering applications.

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